Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Research 341 (2006) 738–743
Carbohydrate RESEARCH

Structural elucidation of the extracellular and cell-wall teichoic acids of *Staphylococcus aureus* MN8m, a biofilm forming strain

Evgueny Vinogradov,[a] Irina Sadovskaya,[b] Jianjun Li[a] and Saïd Jabbouri[b,*]

[a]*Institute for Biological Sciences, National Research Council, 100 Sussex Drive, Ottawa, ON, Canada KIA OR6*
[b]*Laboratoire de Recherches sur les Biomatériaux et Biotechnologies INSERM ERI 002, Université du Littoral-Côte d'Opale, Bassin Napoléon BP 120, F-62327 Boulogne-sur-mer, France*

Received 15 November 2005; accepted 9 January 2006
Available online 3 February 2006

Abstract—Extracellular teichoic acid, an essential constituent of the biofilm produced by *Staphylococcus epidermidis* strain RP62A, is also an important constituent of the extracellular matrix of another biofilm producing strain, *Staphylococcus aureus* MN8m. The structure of the extracellular and cell wall teichoic acids of the latter strain was studied by NMR spectroscopy and capillary electrophoresis–mass spectrometry. Both teichoic acids were found to be a mixture of two polymers, a (1→5)-linked poly(ribitol phosphate), substituted at the 4-position of ribitol residues with β-GlcNAc, and a (1→3)-linked poly(glycerol phosphate), partially substituted with the D-Ala at 2-position of glycerol residue. Such mixture is unusual for *S. aureus*.

Keywords: *Staphylococcus aureus*; Teichoic acid; Biofilm

1. Introduction

Staphylococcus aureus and coagulase-negative staphylococci (CoNS), with *Staphylococcus epidermidis* as a leading species, are recognized as a major cause of nosocomial infections associated with the use of implanted medical devices. These so-called 'chronic polymer-associated infections' are characterized by the ability of the causative microorganisms to colonize surfaces of biomaterials by adhering to their surface in biofilms—structured communities of cells encased in self-produced polymeric matrix. The capacity of *Staphylococci* to form biofilms allowing it to evade host immune defense mechanisms and antibiotic therapy is considered to be crucial in colonizing the surfaces of the implants. An understanding of the composition of staphylococcal biofilms and the detailed chemical structure of biofilm components is essential for a clear understanding of the pathogenesis of these bacteria, and the designing of new therapeutic tools against staphylococcal infections.

We have recently shown that along with the well-characterized poly-β-(1→6)-*N*-acetylglucosamine (PNAG)–polysaccharide intercellular adhesin (PIA), teichoic acid (TA) was an essential constituent of the model biofilm-producing strain *S. epidermidis* RP62A.[1] We elucidated the structures of the cell-wall (CW) and extracellular (EC) TA of this strain. The structures were found to be a (1→3)-linked poly(glycerol phosphate), substituted at the 2-position of glycerol residues with α-Glc, α-Glc*N*Ac and, most interestingly, with α-Glc6Ala.[2]

We have also shown that the extracellular TA is a part of biofilm of a number of clinical strains of CoNS[3,4] and that the chemical composition of EC and CW TAs of the studied strains were similar.[3] Since it is known that, in a number of Gram-positive strains, a large fraction of the CW TA is located in a 'fluffy'-layer region beyond the cell wall,[5] we suggested that in case of biofilm-forming CoNS, the CW TA is partly released from the cell surface into the extracellular space and becomes a part of the extracellular 'slime' in cell aggregates, or a part of a biofilm, when the cell clusters are attached to a solid surface.[3]

* Corresponding author. Tel.: +33 3 21 99 45 17; fax: +33 3 21 99 45 24; e-mail: jabbouri@univ-littoral.fr

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.01.012

In the present study, we have elucidated the chemical structure of an extracellular (EC) TA isolated from a biofilm of another model biofilm-producing strain, *S. aureus* MN8m, which is considered as a PNAG over-producer.[6] We have shown that the EC TA, which is a part of its biofilm, has the same structure as its CW TA. Both TAs were mixtures of two polymers, a (1→3)-linked poly (glycerol phosphate) and a (1→5)-linked poly(ribitol phosphate), present in a similar amount.

2. Results

2.1. Preparation of the EC and CW TAs

S. aureus MN8m was grown in a static culture in Erlenmeyer flasks in TSB, conditions which were shown to be favorable for biofilms production. The biomass, forming a thick amorphous adherent biofilm layer on the bottom of the flasks, was collected, and the extracellular polymers released from the cells by sonication with the minimum cell lysis.[1] The crude extract was concentrated and partly deproteinated by addition of trichloroacetic acid (TCA) to a concentration of 5%, and fractionated on a Sephacryl S-300 column, as described earlier.[1] Similarly to *S. epidermidis* RP62A,[1] the elution profile showed two main peaks: a high molecular weight PNAG and a peak of aminosugar-containing material at lower MW, most probably corresponding to the EC TA (Fig. 1).

Lower MW peak fractions were pooled, de-proteinated by extraction with aq phenol, phenol/$CHCl_3$ and desalted on a Sephadex G-50 column, as described earlier,[2] to give the crude EC TA.

The CW TA was extracted from the sodium dodecyl sulfate-treated cells by extraction with 5% TCA.[2] For detailed structural analysis, the TAs were further purified by ion-exchange chromatography.

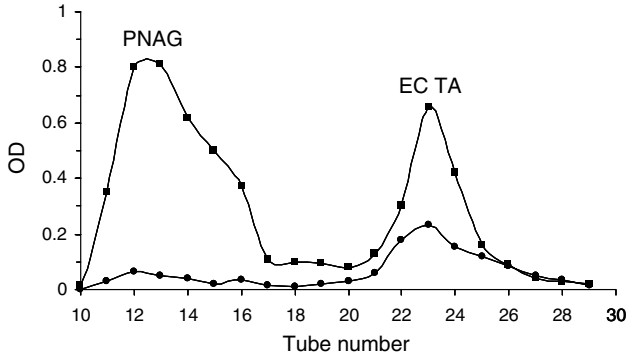

Figure 1. Typical elution profile of the crude extracellular biofilm extract of *S. aureus* MN8m on Sephacryl S-300 column. Aliquots (100 μL) of each 5-mL fraction were assessed for neutral (●, OD_{485}) and aminosugars (■, OD_{530}). Data correspond to 2 l of bacterial culture.

2.2. Structural analysis of TAs

Monosaccharide analysis of both CW and EC TA with 48% HF treatment prior to acid hydrolysis showed the presence of GlcN, glycerol and ribitol. The detailed structure of the CW TA was elucidated by NMR spectroscopy. Its ^{1}H (Fig. 2) and ^{13}C spectra were fully assigned by 2D homonuclear and heteronuclear correlation experiments. Figure 3 shows the HSQC spectrum of the CW TA, and the assignments are summarized in Table 1. The corresponding structures are shown schematically in Figure 4. Spin systems of ribitol (unit **A**), β-GlcNAc (unit **B**), alanine (**Ala**), glycerol (unit **C**), and acylated glycerol (unit **D**) were identified in the spectra. Protons H-1 and H-3 of the glycerol residues and H-1 and H-5 of ribitol residues showed correlation to ^{31}P signal 1.54 ppm (data not shown), indicating the presence of a (1→3)-linked poly(glycerol phosphate) and a (1→5)-linked poly(ribitol phosphate) chains. H-1 of the GlcNAc residue **B** showed NOE correlation to H-4 (H-2) of the ribitol residue **A**, as well as HMBC correlation to C-4 (C-2) of the same residue, indicating that the GcNAc residue was linked to the ribitol at the position 4 (or 2). The structure of the poly(ribitol phosphate) is therefore in a good agreement with the one previously described for *S. aureus* H.[7] Ribitol is symmetrical and positions 2 and 4, as well as 1 and 5, are equivalent. However, biosynthetically it originates from D-ribose, which allows to select correct atom numbering. The choice between the H-2 and H-4 position was made earlier based on the fact that the glyceric acid, obtained from the TA of *S. aureus* H by dephosphorylation, periodate oxidation, oxidation with bromine and

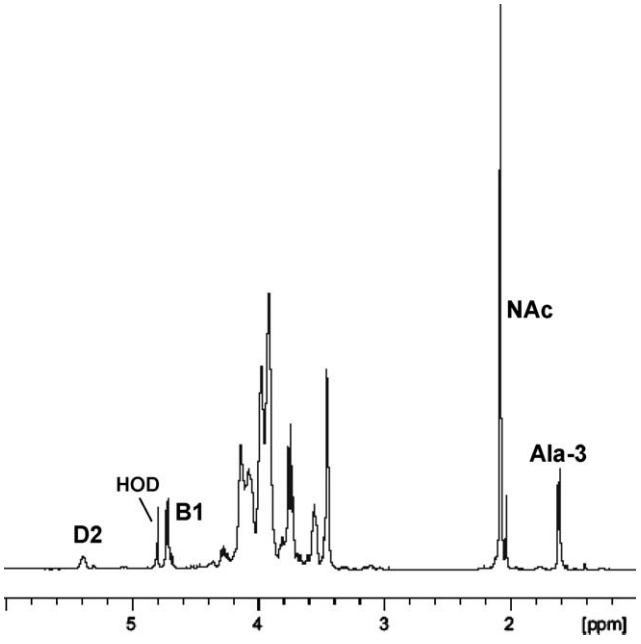

Figure 2. ^{1}H NMR spectrum of the cell-wall TA of *S. aureus* MN8m.

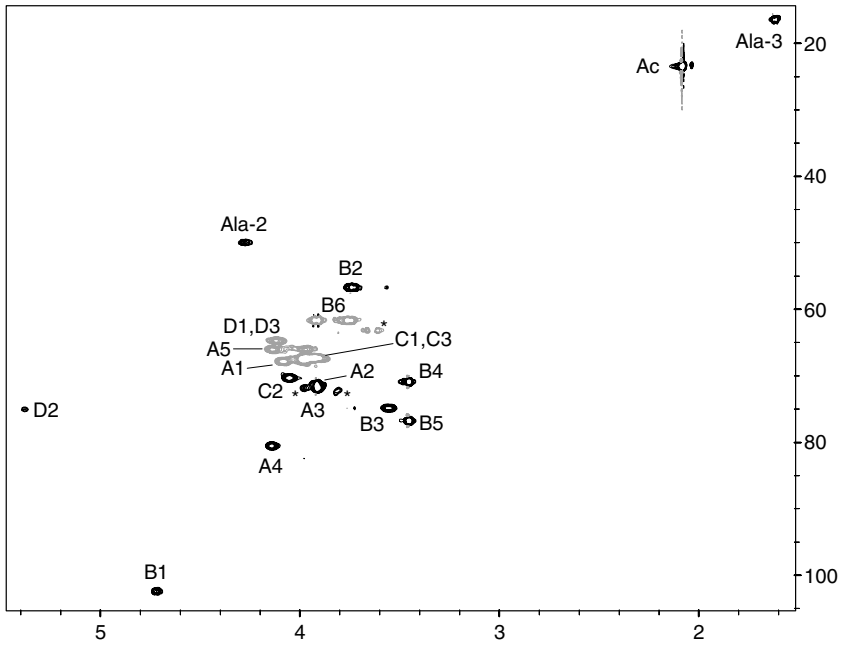

Figure 3. Heteronuclear ^{1}H–^{13}C chemical shift correlation (HSQC) spectrum of the cell-wall TA of *S. aureus* MN8m. Negative cross-peaks (corresponding to the CH_2 groups) appear in grey, and positive cross-peaks (corresponding to the CH and CH_3 groups) appear in black.

Table 1. NMR data

Unit	Nucleus	1/1′	2	3/3′	4	5/5′	6/6′
Ribitol **A**	^{1}H	3.97/4.07	3.91	3.88	4.13	3.96/4.13	
	^{13}C	67.7	71.4	72.1	80.4	66.0	
β-GlcNAc **B**	^{1}H	4.74	3.73	3.56	3.46	3.45	3.75/3.91
	^{13}C	102.2	56.7	75.0	71.0	76.7	61.7
Gro **C**	^{1}H	3.89/3.96	4.04	3.89/3.96			
	^{13}C	67.3	70.5	67.3			
Gro **D**	^{1}H	4.12	5.38	4.12			
	^{13}C	64.6	74.9	64.6			
Ala	^{1}H		4.27	1.62			
	^{13}C	170.9	49.9	16.3			

NAc signals at: C-1 175.9, H-2 2.08, and C-2 23.3 ppm.

hydrolysis had the D-configuration and, consequently, originated from atoms 3–5 of D-ribitol. Thus corresponding glucosaminyl-ribitol was identified as a 4-*O*-(*N*-acetyl-D-glucosaminyl)-D-ribitol.[7] We therefore concluded that the GlcNAc was linked to O-4 of all ribitol residues.

Unlike ribitol residues, which were glycosylated with GlcNAc nearly at 100%, most of glycerol residues were unsubstituted. A minor fraction of glycerol was acylated by Ala residues at O-2, which caused low field shift of its H-2 signal from 4.04 to 5.38 ppm and upfield shift of both C-1 and C-3 from 67.3 to 64.6 ppm (Table 1 and Fig. 3). HMBC correlation was observed between H-2 of acylated glycerol to C-1 of alanine residue, proving acylation of the glycerol at O-2 (data not shown).

A question of whether ribitol and glycerol units are present in one or separate polymeric chains was solved using capillary electrophoresis–mass spectrometry (CE-MS).

Although intact polysaccharides could not be detected, repeating unit information was obtained by employing in-source fragmentation strategy.[8] No fragments containing simultaneously glycerol and ribitol were observed (Table 2), pointing to the presence of separate poly(glycerol phosphate) and poly(ribitol phosphate) chains. Additional proof of this comes from the fact that lower orifice voltage (decluster potential) was required to observe ions originating from poly(glycerol phosphate) chains, thus ruling out the possibility that both chains could be connected at one point. Fragments of poly(ribitol phosphate) chain with ribitol units non-substituted with GlcNAc were also observed, which indicates that not all possible structural components were seen in NMR spectra (Table 2).

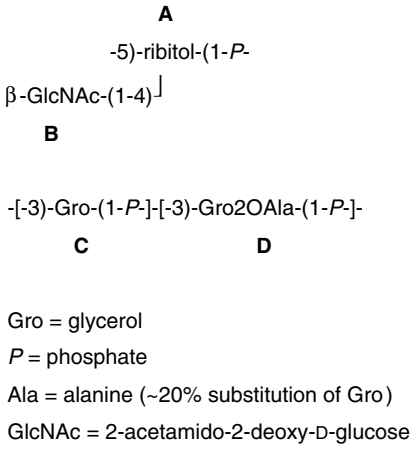

Figure 4. Suggested schematic structure of the CW and EC TA of *S. aureus* MN8m. Glycerol units are substituted with alanine at approx. 20%.

Table 2. Secondary ions observed in mass spectra (observed $m/z =$ [calculated mass − 1] and/or [calculated mass − 1 − H_2O])

Ribitol	GlcNAc	Gro	P	Ala	Mass
1	1		1		435.3
2	2		2		852.6
2	2		1		772.6
2	1		2		649.4
2	1		1		569.5
		3	3		480.2
		4	3		554.3
		3	3	1	551.3
		4	3	1	625.4
		4	4		634.2

NMR and MS analysis of the EC TA showed the presence of the same two polymers. β-GlcNAc substituted 100% of ribitol units in EC TA.

2.3. Comparative analysis of TA and LTA preparations

The best studied *S. aureus* CW TA is a GlcNAc-substituted poly(ribitol phosphate) TA of *S. aureus H*.[7,9] Here, we have shown that TA from *S. aureus* MN8m was a mixture of two polymers—a β-GlcNAc substituted (1→5)-linked poly(ribitol phosphate), a structural characteristic for the TA of *S. aureus*, and a poly(glycerol phosphate), quite unusual for TA of *S. aureus* and occurring mostly in TAs of *S. epidermidis*.[2] On the other hand, the published structure of the lipoteichoic acid (LTA) of *S. aureus* comprises a poly(glycerol phosphate) chain of 28–30 units linked via a diglucosyl (gentobiosyl) unit to diacylglycerol.[10] In order to confirm that the poly(glycerol phosphate) chain was indeed a teichoic acid polymer rather than LTA, the crude TA preparations were subjected to hydrophobic interaction chromatography on Octyl-Sepharose, a method which is largely used for the purification of LTA.[11] No phosphate-containing material was retained on the column; and the not-retained fraction had the same composition as the crude TA. In order to verify the efficiency of the hydrophobic interaction chromatography for the separation of TA and LTA of *S. aureus* MN8m, the LTA was extracted from the defatted cells with aq phenol,[11] purified by the hydrophobic interaction chromatography at the same conditions as the crude TA, and analyzed by GLC.

While crude LTA contained some ribitol, most probably arising from the TA, no ribitol was detected in the purified (retained) fraction of LTA. It contained Gro and Glc in an approximate molar ratio 10:1, in agreement with a published structure.[10] Summarizing the data described above, it can be concluded that the poly(glycerol phosphate) chains indeed belong to the TA of *S. aureus* MN8m.

3. Discussion

It is considered that TA of *S. aureus* is typically a poly(ribitol phosphate), while the polymer chain of its LTA is a poly(glycerol phosphate). Some unusual *S. aureus* strains, however, contain glycerol instead of ribitol TA in their cell walls.[12] We have shown here that in the TA of *S. aureus* MN8m, a poly(glycerol phosphate) chain is present along with the poly(ribitol phosphate). Such mixtures were previously reported for type strains of *S. xylosis* and *S. saprophyticus* group Ib.[12] In these earlier studies, however, only products of HF hydrolysis of TAs were analyzed, and a possible substitution with alanine was not elucidated. In *S. xylosis* and *S. saprophyticus* type strains, ribitol residues were reported to be partially substituted with β-GlcNAc, and glycerol residues—with α-GlcNAc. In the TA of *S. aureus* MN8m, described in the present study, ribitol residues were shown to be fully substituted with β-GlcNAc and did not contain Ala substitution. The (1→3)-linked poly(glycerol phosphate) chains, unlike in the case of *S. epidermidis* RP62A, do not carry sugar substitution but instead, are esterified with D-Ala residues. Interestingly, both glycerol and ribitol have been detected in the CW and EC TAs of a number of clinical biofilm forming staphylococcal strains of our collection,[3] indicating that the presence of two different poly(polyol phosphates) in TAs of *S. aureus* MN8m is not an exception.

Another interesting question is the presence of the TA (both chains) in the biofilm of this biofilm-overproducer. In the recent study, Joyce et al.[13] reported the presence of a polyglycerophosphate component of TA, as a 'common contaminant of *S. aureus* preparations', and apparently forming a non-covalent complex with PNAG.

Specific interactions between the PNAG and the EC TAs could play an important role in the mechanism of the biofilm formation of this PNAG and biofilm-overproducing strain.

4. Experimental

4.1. Preparation of EC and CW TAs

The EC TA was prepared from crude biofilms extract of *S. aureus* MN8m (Harvard Medical School, Boston, MA), grown statically in 700 mL of TSB in a 3-liter Erlenmeyer flask as described in the literature.[1] The medium was discarded, and the adherent biofilms gently washed with saline. Cells and adherent biofilms were then collected in saline with glass beads (diam. ~6 mm). Cell-associated extracellular polymers were detached by sonication on ice (IKASONIC sonicator, 230 V, 50/60 Hz IKA Labothechnik, Germany) at 40% amplitude, cycle 0.4; 4 × 20 s, conditions minimizing cells lysis. Cells were collected by centrifugation and used for the preparation of the CW TA. The crude saline extract was deproteinated by the addition of TCA to a concentration of 5%, concentrated on an Amicon® Ultrafiltration Cell with a 10 kDa molecular cutoff membrane (Millipore) and applied to a Sephacryl S-300 column. Fractions, corresponding to the EC TA were collected and further deproteinated by extraction with water-saturated phenol (3×) and phenol–$CHCl_3$ 1:1 (v/v, 3×), and re-purified on a Sephadex G-50 column. Cells, remaining after the extraction of extracellular polymers, were defatted by boiling with 4% SDS, and the CW TA was extracted with 4% TCA as described in the literature.[2] The crude CW TA was fractionated on Sephadex G-50 column and lyophilized.

Crude CW TA was O-deacylated by treatment with 5% aqueous ammonium hydroxide for 3 h at 37 °C. For NMR and MS analysis, TA samples were further purified by anion exchange on a Hitrap Q column, as described earlier.[2] The crude CW TA (10 mg) was separated by hydrophobic interaction chromatography on Octyl Sepharose 4 Fast Flow column.

Retained and not retained fractions were desalted on G50 and analyzed by GLC (programs a and b) and GLC–MS.

4.2. Preparation of the LTA

For the preparation of the LTA, cells were grown overnight aerobically in TSB medium (700 mL in a 3 l Erlenmeyer flask) with shaking (80 strokes/min), collected by centrifugation (5000*g*, 4 °C, 20 min), washed with 0.9% NaCl, EtOH (3×), acetone (2×) and air-dried. The delipidated cells were suspended in 120 mL of PBS-buffer, 100 mL of 90% (v/v) aq phenol was added and the mixture was vigorously stirred at 70–75 °C for 30 min.[11] The emulsion was cooled on ice, centrifuged (4000*g*, 4 °C, 30 min). The aqueous phase was removed; to the phenol layer combined with the insoluble residue equal volume of PBS buffer was added, and the extraction procedure was repeated. The joined aqueous phase was dialyzed against water and lyophilized, giving 170 mg of crude LTA preparation. Sixty milligrams of this material was dissolved in water and treated with DNAse (Sigma, 0.1 mg/ml) for 2 h at 37 °C. Proteins were extracted with phenol, the water phase dialyzed, freeze-dried, re-dissolved in the 20 mM Na-acetate buffer (pH 4.7) and purified by hydrophobic interaction chromatography on Octyl Sepharose 4 Fast Flow column.

4.3. General and analytical methods

Glycerol, ribitol and monosaccharides were identified by GLC on a Shimadzu GC-14 B gas chromatograph equipped with a flame ionization detector and a Zebron ZB-5 capillary column (30 m × 0.25 mm), with hydrogen as carrier gas, using a temperature gradient 170 °C (3 min), 260 °C at 5 °C/min (program a) or 140 °C (3 min), 260 °C at 10 °C/min (program b). The absolute configuration of alanine was determined by comparison of the GLC retention time of its *N*-acetyl-(*R*)-2-butyl ester with authentic standards.

Prior to analysis, samples of TA were depolymerized by aqueous 48% hydrofluoric acid (HF, Acros Organic) for 48 h at 4 °C. HF was evaporated under a stream of nitrogen, the residue hydrolyzed with 4 M trifluoracetic acid (TFA, 120 °C, 2 h) and converted into alditol acetates by conventional methods. In order to preserve the volatile glycerol acetate, all evaporation to dryness of the alditol acetate samples was avoided.

Gel permeation chromatography was carried out on Sephacel S-300 (1 × 90 cm, Amersham Biosciences) and Sephadex G-50 column (1.6 × 95 cm; Amersham Biosciences), irrigated with water. Fractions (5 mL) were assayed colorimetrically for aldose[14] and aminosugars.[15]

Ion-exchange chromatography was carried out on Hitrap Q anion-exchange column containing 5 mL of Q-Sepharose Fast Flow (Amersham Biosciences) in a gradient of water—1 M NaCl over 1 h and fractions containing TA were identified by charring of the eluate spots on TLC plate with 5% H_2SO_4. The product was desalted by gel permeation chromatography on Sephadex G-50 column.

Hydrophobic interaction chromatography was carried out on a column (1 × 7 cm) of Octyl Sepharose 4 Fast Flow (Amersham Biosciences), equilibrated with 20 mM Na acetate buffer, pH 4.7. The column was washed with the same buffer (10 mL). LTA was eluted with the 50% 1-propanol in the same buffer. Fractions (1 mL) were monitored for OD_{260} and colorimetrically for phosphate[16]. Phosphorus-containing fractions were

pooled, dialyzed (Spectra/Por 6–8 KDa cutoff membrane) and lyophilized.

Mass spectrometry: All MS experiments were performed as previously described.[2]

NMR spectroscopy: NMR spectra were recorded at 25 °C in D_2O on a Varian UNITY INOVA 500 instrument, using acetone as reference (^1H, δ 2.225 ppm, ^{13}C, δ 31.5 ppm). Varian standard programs COSY, NOESY (mixing time of 200 ms), TOCSY (spinlock time 120 ms), HSQC, and gHMBC (evolution delay 100 ms) were used. ^1H–^{31}P HMQC correlation spectra were recorded on Varian UNITY INOVA 400 instrument.

Acknowledgements

This work was supported by Contract Plan Etat-Region (CPER) of the Nord-Pas de Calais Region and INSERM (France). The authors thank Prof. P. Hardouin for his support. The authors are grateful to Prof. G. Pier, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, for providing the bacterial strain *S. aureus* MN8m.

References

1. Sadovskaya, I.; Vinogradov, E.; Flahaut, S.; Kogan, G.; Jabbouri, S. *Infect. Immun.* **2005**, *73*, 3007–3017.
2. Sadovskaya, I.; Vinogradov, E.; Li, J.; Jabbouri, S. *Carbohydr. Res.* **2004**, *339*, 1467–1473.
3. Kogan, G.; Sadovskaya, I.; Chaignon, P.; Chokr, A.; Jabbouri, S. *FEMS Microbiol. Lett.* **2006**, *255*, in press.
4. Sadovskaya, I.; Chaignon, P.; Kogan, G.; Chokr, A.; Vinogradov, E.; Jabbouri, S. *FEMS Immunol. Med. Microbiol.*, in press.
5. Neuhaus, F. C.; Baddiley, J. *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 686–723.
6. Maira-Litran, T.; Kropec, A.; Abeygunawardana, C.; Joyce, J.; Mark, G., 3rd.; Goldmann, D. A.; Pier, G. B. *Infect. Immun.* **2002**, *70*, 4433–4440.
7. Baddiley, J.; Buchanan, J. G.; Hardy, F. E.; Martin, R. O.; Rajbhandary, U. L.; Sanderson, A. R. *Biochim. Biophys. Acta* **1961**, *52*, 406–407.
8. Li, J.; Wang, Z.; Altman, E. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1305–1314.
9. Baddiley, J.; Buchanan, J. G.; Martin, R. O.; Rajbhandary, U. L. *Biochem. J.* **1962**, *85*, 49–56.
10. Duckworth, M.; Archibald, A. R.; Baddiley, J. *FEBS Lett.* **1975**, *53*, 176–179.
11. Iwasaki, H.; Shimada, A.; Ito, E. *J. Bacteriol.* **1986**, *167*, 508–516.
12. Endl, J.; Seidl, H. P.; Fiedler, F.; Schleifer, K. H. *Arch. Microbiol.* **1983**, *135*, 215–223.
13. Joyce, J. G.; Abeygunawardana, C.; Xu, Q.; Cook, J. C.; Hepler, R.; Przysiecki, C. T.; Grimm, K. M.; Roper, K.; Yu Ip, C. C.; Cope, L.; Montgomery, D.; Chang, M.; Campie, S.; Brown, M.; McNeely, T. B.; Zorman, J.; Maira-Litran, T.; Pier, G. B.; Keller, P. M.; Jansen, K. U.; Mark, G. E., III *Carbohydr. Res.* **2003**, *338*, 903–922.
14. Dubois, M.; Gilles, K. A.; Hamilton, J. F.; Rebers, P. A.; Smyth, F. *Anal. Biochem.* **1956**, *28*, 350–356.
15. Enghofer, E.; Kress, H. *Carbohydr. Res.* **1979**, *76*, 233–238.
16. Chen, P. S.; Toribara, T. Y.; Warner, H. *Anal. Chem.* **1956**, *28*, 1756–1758.